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Cell Biology

Establishment of clonal human placental cells synthesizing human choriogonadotropin

(transformation by simian virus 40)

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ABSTRACT **Seven clonal human placental cell lines were established by transformation of human first-trimester placental cells with simian virus 40. These transformed cells synthesized native human choriogonadotropin (chorionic gonadotropin) (hCG) as well as the free α and β subunits of hCG. The amount of native hCG synthesized by these cells was, however, lower than the amount of free β subunit. (Both hCG and the β subunit are detected by the radioimmunoassay for β subunit, but only hCG is detected by the radioreceptor assay.) The α and β subunits produced by these transformed placental cells were heterogeneous in size; the sizes of the predominant α and β species, however, corresponded to those of urinary α and β subunits, respectively. The seven cell lines transformed by simian virus 40 had chromosome numbers from the near diploid to the near tetraploid range. Fluorescent staining demonstrated the Y chromosome in all the transformants. Furthermore, B-type glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ 1-oxidoreductase; EC 1.1.1.49) was present in all seven lines. These characteristics ruled out possible HeLa contamination of the transformed lines. Regulation of the synthesis of α and β subunits plus hCG in these transformed human placental cells differed from the regulation in choriocarcinoma cells.**

The human placenta is a complex multifunctional endocrine organ that supports fetal survival and development. Placental functions *in vitro* have usually been studied by using explants and primary cultures, which are difficult to establish and cannot be maintained for a long time in tissue culture. Synthesis of placental hormones by explants or primary cultures declines because the cells dedifferentiate in culture. Choriocarcinoma cells, malignant cells derived from the placenta, have been used extensively as a model system to study placental functions (1–4). These cells do not, however, control the synthesis of certain proteins as does the normal placenta. The level of human choriogonadotropin (hCG) often is higher in patients with choriocarcinoma than it is in women during normal pregnancy (5).

hCG is a glycoprotein tropic hormone normally synthesized by the placenta; it is composed of two nonidentical subunits, (hCGα) α and β (hCGβ) (6). Although the synthesis of hCG and its subunits normally occurs in the placenta, ectopic synthesis has been observed in many nontrophoblastic tumors or tumor-derived cell lines (7–10). The regulation of the synthesis of hCG in trophoblastic tumors differs from that in nontrophoblastic tumors (11). Since the regulation of hCG synthesis in choriocarcinoma cells may differ from that in normal placenta, it is necessary to establish another type of placental cell to study placental functions. Human placental cells transformed by simian virus 40 (SV40) have therefore been established. These transformants, like choriocarcinoma cells, produce placental proteins. The control mechanisms for the synthesis of hCG differs in the SV40-transformed placental cells and the choriocarcinoma cells.

MATERIALS AND METHODS

Cells and Culture Conditions. Human first-trimester placenta (subterm placenta) was minced and digested with collagenase (0.1%, Worthington Biochemical Co., Freehold, NJ) for 20 min at 37°. The digest was centrifuged, and the cell pellet was resuspended in α modified minimal essential medium supplemented with 10% fetal bovine serum (Flow Laboratories, Rockville, MD), streptomycin (100 μg/ml), and penicillin (100 units/ml). These primary subterm placental cells, designated "SP" cells, were used directly for transformation.

Transformation. After primary SP cells had been in culture for 4 days, they were infected with *tsA255* virus (12), a temperature-sensitive mutant of SV40 (multiplicity of infection = 10). After the virus was allowed to adsorb for 2 hr at 33°, minimal essential medium containing 10% fetal bovine serum was added to the infected flask, and the cells were incubated at 33°. After 24 hr of incubation, the cells were treated with trypsin and serially diluted. The medium was replaced twice weekly. Transformed colonies could be identified by 5 weeks after infection. Seven independent transformants were established from the same first-trimester placenta.

Chromosome Analysis. The presence of the human Y chromosome was determined by fluorescent staining (13), and trypsin-Giemsa banding of chromosomes (14).

Glucose-6-Phosphate Dehydrogenase. The electrophoretic mobility of the glucose-6-phosphate (G6*P*) dehydrogenase (D-glucose-6-phosphate:$NADP^+$ 1-oxidoreductase; EC 1.1.1.49) species in SV40-transformed placental cells was determined by Cellogel electrophoresis as described (15). Normal A- and B-type G6*P* dehydrogenase were used as standards.

SV40 T-Antigen. The presence of T-antigen in the transformed placental cells was determined by the immunofluorescence method of Anderson and Martin (16). Anti-T serum, lyophilized fluorescein-conjugated anti-hamster gamma globulin, and rhodamine contrast stain, prepared by Huntingdon Laboratories (Brooklandville, MD), were obtained from the National Cancer Institute.

Radioimmunoassays and Radioreceptor Assay. All radioimmunoassays were done by the double-antibody technique described by Rosen and Weintraub (8). Antisera used were: anti-hCG serum (H80) (17), anti-hCGβ serum (Sb6) (18), and

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Abbreviations: hCG, human choriogonadotropin (chorionic gonadotropin); SV40, simian virus 40; Con A, concanavalin A; G6*P* dehydrogenase, glucose-6-phosphate dehydrogenase; Bt_2cAMP, dibutyryl cyclic AMP.

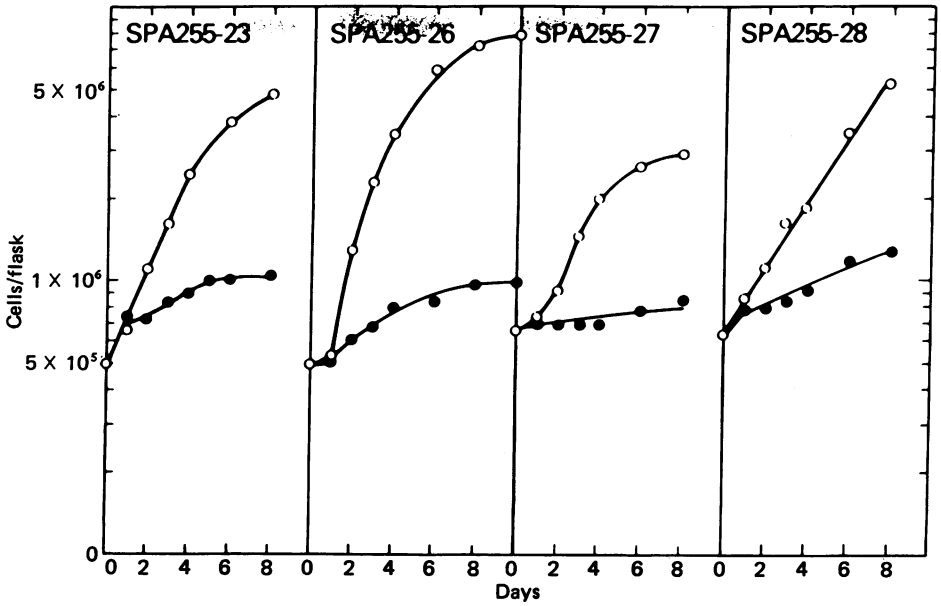

FIG. 1. Growth of transformed human placental cells. Cells were grown in 25-cm² flasks, and medium was changed every other day. Cells were counted with a Celloscope 112TH (Particle Data, Inc., Elmhurst, IL). ○, 33°; ●, 40°.

anti-hCGα serum (CA3) (11). Purified preparations of hCG (CR119, 11,600 IU/mg, ventral prostate weight assay, Second International Standard hCG) and of hCGα (CR117) were radioiodinated and used as standards and tracers. hCG (CR119) had 1–3% crossactivity in the hCGα assay; hCGα had 0.02–0.05% crossreactivity in the hCG assay. Complete medium not exposed to cells had no detectable hCG, hCGβ, or hCGα. Antisera, standard hCG, hCGβ, and hCGα were kindly provided by K. Catt, G. Hodgen, and H. Chen.

The radioreceptor assay for hCG was performed by the method of Catt *et al.* (19), with a crude rat testis homogenate as the source of receptor.

RESULTS

Establishment and Transformation of Human Placental Cells. Five weeks after infection of primary human first-trimester placental cells (SP cells) with SV40 *tsA255* virus, clones of transformed cells could be identified. Seven lines of transformed placental cells were established from the same placenta. The primary SP cells could be maintained in culture for a few weeks, but could not be subcultured. The SV40-transformed cells proliferated in culture, formed multilayered sheets, and grew to high cell densities at the permissive temperature (33°; Fig. 1). At the nonpermissive temperature (40°), the transformed cells behaved like normal placental cells: they grew slowly and to lower saturation densities (Fig. 1) and they could not overgrow normal placental monolayers. These transformed cells therefore have the advantage of behaving like normal cells (at 40°), but not the disadvantages of primary placental cells, which dedifferentiate in culture.

These transformed placental cells shed SV40; anti-SV40 serum (1%, Flow Laboratories, Rockville, MD) was therefore routinely added to the culture media to inhibit plaque formation.

In nearly 100% of the cells, T-antigen was clearly localized within the nucleus, as revealed by the immunofluorescence technique.

Biosynthesis of hCG, hCGα, and hCGβ. All seven transformed human placental cell lines synthesized materials reacting with antisera to hCGα and to hCGβ (Fig. 2). The anti-hCGα serum measures hCGα, and the anti-hCGβ serum measures both hCGβ and hCG. The amounts of hCGα and hCGβ plus hCG synthesized varied among the cells. JEG-3 choriocarcinoma cells synthesized more of these proteins than any of the SV40-transformed lines.

Over 90% of the hCGα and hCGβ plus hCG in the culture medium was adsorbed to a column of (concanavalin A) Con A-Sepharose (Fig. 3A).

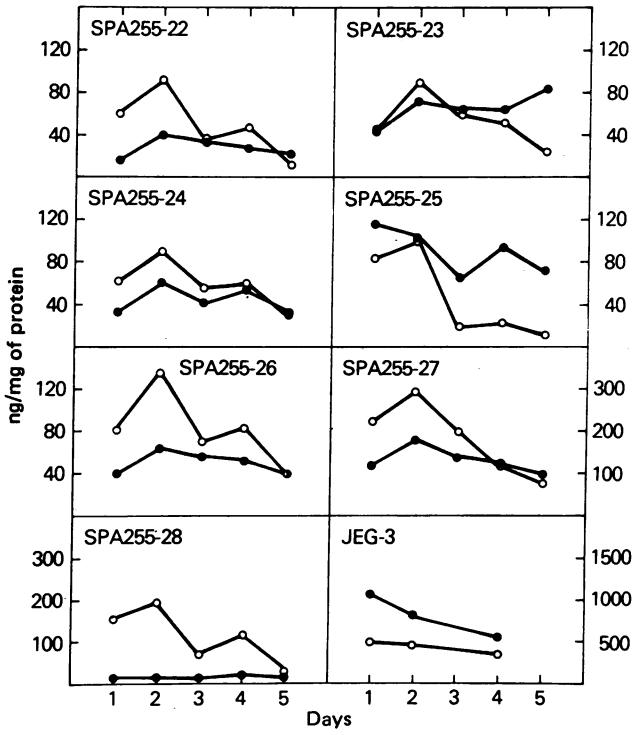

FIG. 2. Synthesis of hCGβ plus hCG and hCGα by clonal strains of transformed human placental cells. Cultures were grown in 25-cm² flasks at 33°, and medium was changed every other day. Day 0 was 1 day after plating. At the indicated times, medium from each flask was assayed for hCGβ plus hCG and separately assayed for hCGα. JEG-3 cells are choriocarcinoma cells established by Kohler and Bridson (3). ○, hCGβ plus hCG; ●, hCGα.

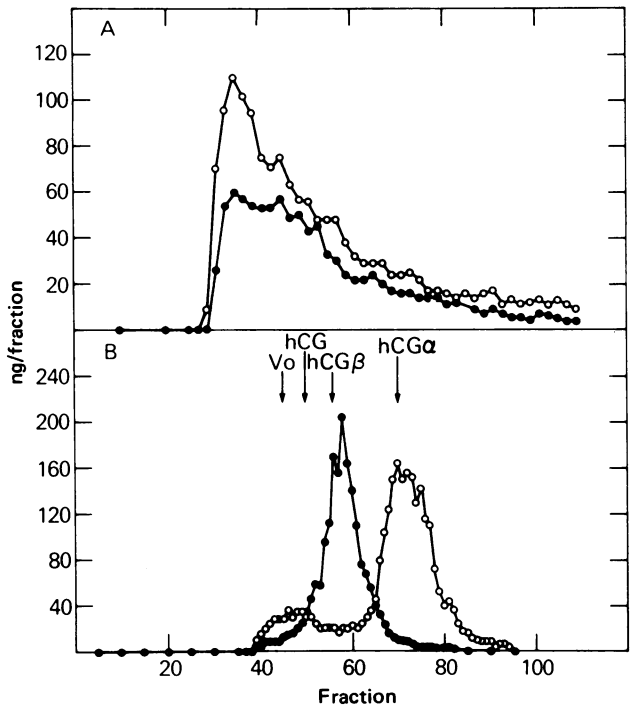

FIG. 3. (*A*) Con A-Sepharose column chromatography of hCG in culture media from SPA255-27 cells. hCG and its subunits were purified from culture medium with Con A-Sepharose (Pharmacia, Sweden) that had been equilibrated with Dulbecco's balanced salt solution supplemented with 1 mM $MgCl_2$, 1 mM $CaCl_2$, and 1 mM $MnCl_2$. The medium plus Con A-Sepharose was stirred overnight at 4° and was poured into a 2.4 × 20 cm column. The column was washed with phosphate-buffered saline and the bound proteins were eluted with phosphate-buffered saline containing 0.5 M methyl-α-D-glucopyranoside. The flow rate was 65 ml/hr, and the fraction size was 3 ml per tube. (*B*) Sephadex G-100 chromatographic patterns of hCG produced by SPA255-27 cells. Fractions 27–90 from the Con A-Sepharose column were pooled, concentrated, and applied to the Sephadex G-100 column (1.5 × 125 cm) equilibrated with phosphate-buffered saline at 4°. The fraction size was 2 ml per tube. The arrow marked V_0 indicates the void volume. The migration positions of hCG, hCGβ, and hCGα in this column were determined separately and are indicated by the respective arrows. ○, hCGα; ●, hCGβ plus hCG.

The immunoreactive proteins synthesized by these cells are, therefore, glycoproteins, as are urinary hCG and its subunits. The glycoproteins eluted from the Con A-Sepharose column were measured for hCG receptor-binding activity by the radioreceptor assay (Fig. 4*A*). The slope of the dose-response curve for the material eluted from Con A-Sepharose was indistinguishable from that for urinary hCG. The receptor-active material, hCG, represented only 20% of the hCGβ plus hCG estimated by the radioimmunoassay.

The hCGα and hCGβ plus hCG from Con A-Sepharose were subjected to gel filtration on a Sephadex G-100 column (Fig. 3*B*). The majority of the hCGβ plus hCG immunoreactive material coeluted with hCGβ. The hCGβ produced by the transformants was heterogeneous in size; the main hCGβ peak eluted slightly behind the purified urinary hCGβ. The hCGα produced by the transformants was also heterogeneous in size; the main peak, however, coeluted with urinary hCGα. In addition to the hCGα in the main peak, hCGα-immunoreactive material appeared also as a broad peak at the void volume of the column. The nature of this material is not known; it is, however, possible that it represents the precursor of hCGα.

Comparison of hCGα and hCGβ Purified on Sephadex G-100 with Urinary hCG and Its Subunits. The immunological determinants of the proteins synthesized by the transformed placental cells were compared with the urinary hCG and its subunits (Fig. 4). The slope of dose-response curve for the hCGα purified from SPA255-27 medium was indistinguishable from that for urinary hCGα (Fig. 4*B*). The slopes of dose-response curves for the hCGα-like material in unfractionated media from all seven transformants were also indistinguishable from that for urinary hCGα. The dose-response curve for hCGβ purified from SPA255-27 medium paralleled the inhibition line of urinary hCGβ in the presence of either anti-hCG–antiserum H80 (Fig. 4*C*) or anti-hCGβ–antiserum Sb6 (Fig. 4*D*). The dose-response curve for the purified hCGβ did not parallel the inhibition line of native hCG.

Chromosome Studies and G6*P* Dehydrogenase Typing. The seven SV40-transformed cell lines (SPA255-22 to 28) had chromosome numbers from the near diploid to the near tetraploid range. All lines had some cells with broken, fragmented, minute, dicentric, or ring chromosomes; the incidence of such abnormal chromosomes varied from 12% of the cells in SPA255-28 to 50% of the cells in SPA255-24. Fluorescent staining demonstrated two Y-bodies (Fig. 5*A*) in all the tetraploid range cells and one Y-body (Fig. 5*B*) in all the diploid range cells. The G6*P* dehydrogenase in all seven placental lines was the B type, as demonstrated by Cellogel electrophoresis. These characteristics ruled out possible HeLa contamination of the transformed lines.

Regulation of Synthesis of hCG and hCGα by Sodium Butyrate, Dibutyryl Cyclic AMP, and 5-Bromo-2′-deoxyuridine in SV40-Transformed Placental Cells. The regulation of the synthesis of hCG and hCGα is different in trophoblastic and nontrophoblastic tumors (11; J. Y. Chou, S.-S. Wang, and J. C. Robinson, unpublished observations). In choriocarcinoma cells sodium butyrate and 5-bromo-2′-deoxyuridine (BrdUrd) inhibited the synthesis of hCG and hCGα, whereas dibutyryl cyclic AMP (Bt_2cAMP) stimulated synthesis. In nontrophoblastic tumor cells, however, sodium butyrate induced the syntheses of both hCG and hCGα, and BrdUrd induced hCGα synthesis. Although Bt_2cAMP induced the synthesis of hCG and hCGα in nontrophoblastic tumor cells, cyclic AMP and 8-bromo-cyclic AMP did not affect the synthesis of either peptide in HeLa S_3 cells.

Choriocarcinoma cells are characterized by abnormally high hCG production (5). It is possible that the regulation of hCG synthesis in these cells differs from that of placenta *in vivo*. The effects of sodium butyrate, Bt_2cAMP, and BrdUrd on the synthesis of hCGβ plus hCG and hCGα in the SV40-transformed human placental cells were therefore investigated (Table 1). The SV40-transformed cells increased their synthesis of hCGβ plus hCG and hCGα in the presence of sodium butyrate, in contrast to choriocarcinoma cells. hCGβ plus hCG was induced to a greater extent by sodium butyrate, however, than was hCGα. Although Bt_2cAMP induced the synthesis of hCGα in these transformed cells, it did not affect the synthesis of hCGβ plus hCG. BrUrd, on the other hand, did not affect the synthesis of either peptide in these cells. The regulation of the syntheses of hCG and hCGα, therefore, differed in the two types of placental cells.

DISCUSSION

The appearance of placental proteins in nontrophoblastic tumors indicates a radical change in the control mechanisms for the synthesis of these proteins after transformation (7–10, 20, 21). Indeed, the presence of these placental proteins can provide potential markers for neoplasia (10). To understand the regulation of the synthesis of these proteins in neoplastic cells, it is

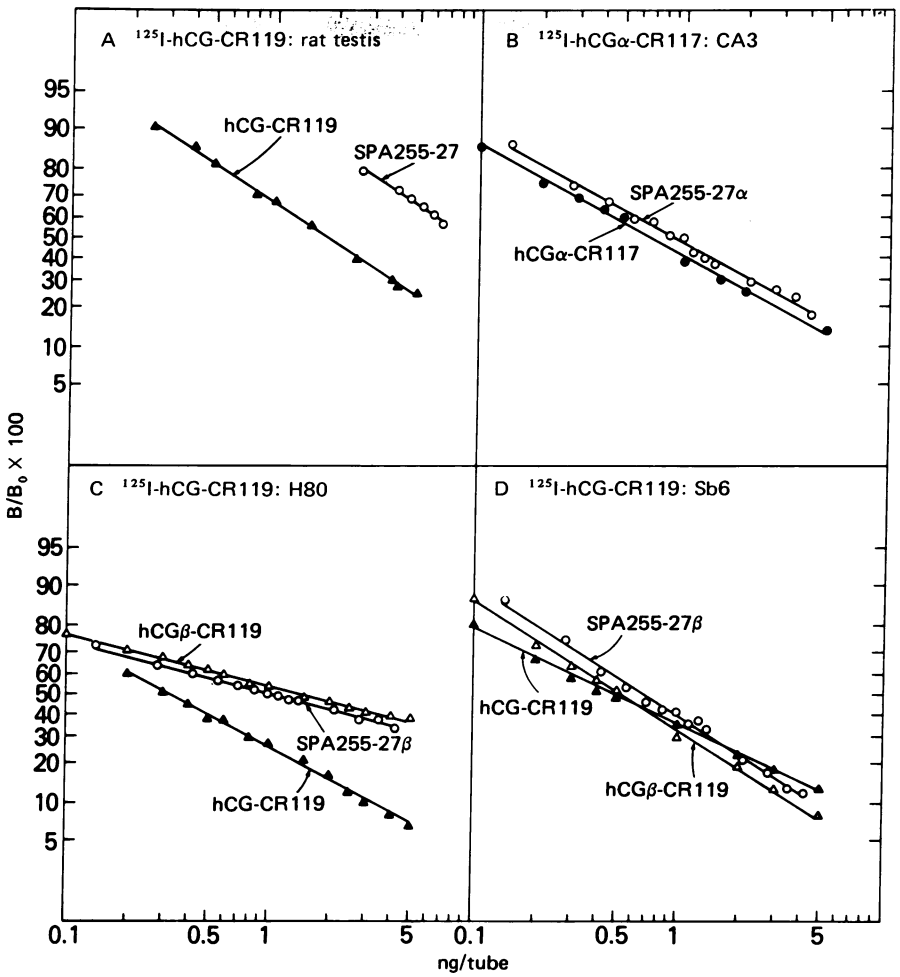

FIG. 4. Dose-response curves of hCG, hCGα, and hCGβ synthesized by the transformed placental cells in a radioreceptor assay or in a radioimmunoassay system.

necessary to have a valid control system in which to study normal placental functions. Normal placental cells (explants and primary cultures) are not good models for the placenta *in vivo* because they are difficult to establish and maintain in culture and because they dedifferentiate gradually. Neither are choriocarcinoma cells a good model. These malignant cells, derived from the cancer of the placenta, grow well and have been used for the past decade as a model system for the study of placental functions (1–4). Cultured choriocarcinoma cells would not, however, be expected to control the synthesis of hCG as does the placenta *in vivo*, since the levels of hCG in choriocarcinoma patients tend to be abnormally high (5).

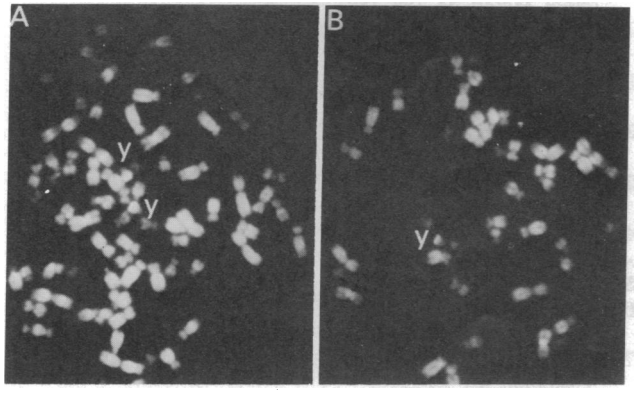

FIG. 5. Fluorescent staining of Y chromosome in (*A*) tetraploid and in (*B*) diploid human placental cells transformed by SV40.

A suitable model system has been developed by the transformation of normal human placental cells with a temperature-sensitive mutant of SV40. These transformed placental cell lines synthesized the placental glycoprotein tropic hormone, hCG, and its α and β subunit, which resembled urinary hCG, hCGα, and hCGβ by the following criteria. First, they are glycoproteins, as demonstrated by their ability to bind Con A-Sepharose. Con A, a hemagglutinating protein isolated from jack bean, forms insoluble complexes with polysaccharides and glycoproteins (22). Second, both hCGα-like and hCGβ-like material produced by the transformants cochromatographed with urinary hCGα and hCGβ on a Sephadex G-100 column. Both the hCGα and hCGβ produced by the transformed cells were, however, heterogeneous populations of molecules. Third, the slopes of dose-response curves for the hCGα-like materials were indistinguishable from that for urinary hCGα. Likewise, the curve for hCGβ-like material was indistinguishable from that for urinary hCGβ. Fourth, native hCG synthesized by the transformants was biologically active and its immunologic determinants were similar to those of urinary hCG.

Several laboratories have demonstrated the heterogeneity of glycoprotein hormones and their subunits (23–25). The heterogeneity of the hCGα and hCGβ produced by the transformants might be due to variations in their carbohydrate moieties. In addition, the existence of a large immunologic

Table 1. Effect of Bt_2cAMP, BrdUrd, and sodium butyrate on the synthesis of hCGα and hCGβ plus hCG in SV40-transformed human placental cells

Cell lines	Control		Bt_2cAMP		BrdUrd		Sodium butyrate	
	hCGα	hCGβ +hCG	hCGα	hCGβ +hCG	hCGα	hCGβ +hCG	hCGα	hCGβ +hCG
SPA255-22	29	46	359	49	42	28	76	167
SPA255-23	63	53	434	71	61	41	86	635
SPA255-24	54	59	224	57	72	37	78	118
SPA255-25	94	23	960	53	146	29	159	553
SPA255-26	53	83	190	62	47	64	92	398
SPA255-27	124	117	1,550	112	168	111	114	1,571
SPA255-28	20	117	219	48	27	89	40	671
JEG-3	622	1,606	12,376	20,957	396	727	478	449

Cultures were exposed to Bt_2cAMP (2 mM), BrdUrd (10 μg/ml), or sodium butyrate (2 mM) for 4 days at 33°. Medium was changed every 2 days. Values given are ng/mg of protein.

species of hCGα in the placenta has been described (23, 25, 26). It is possible that the large hCGα-like material produced by the transformed placental cells was the precursor form of hCGα, as speculated by Weintraub *et al.* (27). Although relatively large quantitites of hCGα and hCGβ were synthesized by these cells, little native hCG was produced. The lack of native hCG might reflect structural defects in the subunits.

Data presented here demonstrate that the regulation of the synthesis of hCGα and hCGβ plus hCG in these transformed placental cells differed from the regulation in choriocarcinoma cells. Although these results have not demonstrated unequivocally that the transformed placental cells more closely approximate normal placenta than do choriocarcinoma cells, available data support this suggestion. First, hCG synthesis in choriocarcinoma patients may be regulated abnormally, leading to the very high levels of hCG observed in many of these patients (5). Second, the transformed placental cells described here are induced by a *tsA* mutant of SV40; the *A* function of SV40 is required for the maintenance of the transformed phenotype in these cells. These cells could assume either a normal (at 40°) or a transformed (at 33°) phenotype; nevertheless, regulation of the synthesis of hCGβ plus hCG and hCGα by sodium butyrate, Bt_2cAMP, and BrdUrd was the same at either temperature. Third, the preservation of the original diploid chromosome number in many of these lines indicates that these cells are closer to normal placental cells than are the aneuploid choriocarcinoma cells. Fourth, SV40-transformed term placental cells synthesize less hCGβ plus hCG than do the transformed subterm placental cells described here. The amounts of hCGβ plus hCG synthesized by the SV40 transformants from subterm and term placental cells were proportional to the amounts of these proteins synthesized *in vivo* by placentas at their respective periods of gestation. Therefore, the transformation of the placental cells by SV40 may not have interfered with the normal control mechanisms for the synthesis of hCGβ plus hCG.

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